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Review

Vitrification of buffalo oocytes and embryos

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A B S T R A C T

During the past decade, vitrification has been acknowledged as an efficient alternative to traditional slow-rate freezing in both human and animal embryology. The buffalo is the major milk and meat producing farm animal in many developing countries. Cryopreservation of buffalo oocytes and embryos is very important in preserving this species for future use. This review discusses the recent buffalo oocytes and embryos vitrification procedures, different types of cryoinjuries, and other factors affecting the vitrification of buffalo oocytes and embryos.

1. Introduction

Cryopreservation of oocytes is very important in preserving female gametes for future use, which permits female genetic material to be stored unfertilized until the appropriate mate can be selected. Moreover, embryo cryopreservation allows the conservation of the full genetic complement of sire and dam and thus has enormous potential for protecting and managing species population integrity and heterozygosity.

Nowadays, buffalo is the major milk and meat producing farm animal in many developing countries. Successful buffalo breeding highly depends on the genetic improvement that can be achieved by the application of assisted reproductive technologies. Although, methods of reproductive biotechnology have been applied in this species, most of them are not as efficient as in bovine. In addition, buffaloes were reported to have poor response to superovulation treatments [1,2] thus manifesting a relatively low yield of in vivo–derived embryos. Hence, it is imperative to study the factors necessary to improve the success rate of the application for reproductive biotechnologies in this species. Efficient oocyte and embryo cryopreservation protocols will widen and improve the strategic implementation of reproductive technologies in buffalo species. During cryopreservation, various types of injury may occur. Among the most damaging is the formation of intracellular ice. The first strategy to prevent intracellular ice formation was to use a lower concentration of cryoprotectant (CPA) and a long slow-cooling stage. This slow-freezing method has been proven to be effective for the cryopreservation of embryos in a wide range of mammalian species. With slow freezing, however, it is difficult to eliminate injuries occurring from ice formation completely. Furthermore, the slow-freezing method requires a long period of time before embryos are stored in liquid nitrogen (LN_2). An alternative form of cryopreservation is vitrification. Moreover, oocytes and embryos of species with typically high lipid content (such as pigs and buffaloes) suffer the loss of membrane integrity or developmental competence during the cooling procedure before the phase transition of the freezing solution due to chilling injuries and osmotic shock.

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Vitrification is defined as "the solidification of a solution brought about not by crystallization but by extreme elevation in viscosity during cooling" [3]. The rapid cooling process can minimize chilling injury and osmotic shock to the embryos. Because of the high intracytoplasmic lipid content, buffalo oocytes are supposed to be particularly sensitive to chilling injuries [4]. It has been reported that slow freezing is not suitable for immature buffalo oocytes, as proven by both poor maturation rates and development to morulae [5]. Correspondingly, vitrification was proven to be more effective than slow freezing for the cryopreservation of *in vitro*–matured buffalo oocytes [6].

Both slow freezing and vitrification technique [7] are used for buffalo embryo cryopreservation, and pregnancies from slow freezing and vitrified [8] embryos, as well as live calves from slow-freezing [9] and vitrified–warmed embryos [10], have been reported. However, a comparison between slow freezing and open-pulled straw (OPS) vitrification of zona-free cloned buffalo embryo found that vitrification was better than slow freezing in terms of the cryosurvival rate [11]. It is proposed that vitrification will definitely become the most suitable method for cryopreservation of any cells and tissues in the near future. This is especially true to buffalo oocytes which are extremely large single cells containing an excess amount of intracellular lipid which makes them very sensitive to cryopreservation. Therefore, vitrification technologies have been applied on buffalo oocytes and embryos more success than slow-cooling methods [11]. However, the developmental competence of buffalo oocytes and embryos is still greatly compromised by vitrification which suggests and the perfect protocol has not yet been established. The purpose of this article is to review the current status of buffalo oocyte and embryo vitrification and the recent development regarding cryoinjuries and poor survival rate.

2. Cryoinjuries of buffalo oocytes and embryos

The major problem with oocyte cryopreservation is low survival and/or poor developmental competence. The primary site of cryopreservation-induced damage is known to be the oocyte membrane. The loss of membrane integrity during chilling and phase transition means the death of the cell by a process resembling necrosis. Further problems associated with chilling and freezing have been reported in mammalian oocytes surviving vitrification including abnormal spindle associated with disorganized microtubules and chromosomes [12,13], altered distribution of cortical granules and increased polyspermy or on the contrary, zona pellucida hardening by premature cortical granule exocytosis impairing fertilization [14]. Mitochondria alterations, poorly developed desmosomes, and lack of tight junctions were identified being more severe in *in vitro*–produced bovine embryos after cryopreservation [15,16].

A crucial step for the success of vitrification is the exposure of oocytes or embryos to the vitrification solution before plunging in LN_2 (or super-cooled air). The major cryoinjuries are the results of ice-crystal formation, solution effects, and osmotic shock. To prevent intracellular ice forming, the efficient uptake of permeating CPA by the cells is desirable which can be achieved by a longer period of exposure to CPA. Conversely, the cells may suffer the loss of viability by the toxicity of the CPA before enough CPA can permeate inside the embryos or oocytes. Optimization of vitrification protocols include (1) reducing container volumes and/or increasing the thermal gradient [17] to increase cooling and/or warming rates, (2) the use of CPA with high permeability but low toxicity, or (3) by supplementation of medium with various additives which increase cry-tolerance of cells.

Vitrification devices, CPA application, quality and source of oocytes and embryos, and exposure time in vitrification solution are the main points affecting vitrification outcomes. Other factors such as: composition of holding medium and temperature of incubation or room temperature and of rehydration, mode of addition, and dilution of CPA are also factors that affect the oocyte and embryo vitrification.

3. Vitrification of buffalo embryos

Conventional cryopreservation by slow freezing of buffalo embryos has been unsuccessful, possibly because multicompartmental structure and low water permeability lead to intracellular ice formation and chilling injury.

Currently, the most widely used protocol applied to any embryo stage is the two-step equilibration in a combination of permeating CPAs, most often ethylene glycol (EG) and dimethyl sulfoxide (DMSO). This approach was reported to be effective for the in-straw vitrification of buffalo embryos with good efficiency in terms of *in vitro* survival rate [18]. However, to date, many protocols and devices have been applied for the vitrification of buffalo embryos with various results as shown in Table 1.

Early stage embryos are more sensitive to vitrification compared with advanced stage embryos. In buffalo, Hufana-Duran et al. [10] estimated the hatching rate from vitrified-thawed embryos at the morula, early blastocyst, blastocyst, and expanded blastocyst stages, showing no differences (75%–90%). In contrast, other authors reported lower cryosurvival rates of buffalo morula stage embryos compared with blastocyst stage embryos [21,22]. To date, several devices have been applied for buffalo embryo vitrification including French straw [10], Cryotop [19], OPS [11]. French straw were popular used in buffalo embryo vitrification at the early stage. Hufana-Duran et al. [10] reported high-hatching blastocyst rate (90%) and birth of live calves following vitrification of buffalo embryos in French straw. When Cryotop was applied, buffalo SCNT blastocysts were more tolerant to vitrification than bovine SCNT blastocysts as indicated by the high survival rate [19]. In a recent report, OPS was found to be superior to slow freezing for the cryopreservation of zona-free cloned buffalo blastocysts based on the improved cry survival rates by the OPS method [11].

There are two sort of CPA as follows: permeating and nonpermeating, which are added in cryopreservation solution to avoid ice formation and protect cellular organelles. Permeating CPA are small molecules that readily penetrate the membranes of cells, form hydrogen bonds with intracellular water molecules and lower the freezing